

# Molecular and Functional Characterization of Broccoli *EMBRYONIC FLOWER 2* Genes

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**Polycomb group (PcG) proteins regulate major developmental processes in Arabidopsis. *EMBRYONIC FLOWER 2* (*EMF2*), the VEF domain-containing PcG gene, regulates diverse genetic pathways and is required for vegetative development and plant survival. Despite widespread *EMF2*-like sequences in plants, little is known about their function other than in Arabidopsis and rice. To study the role of *EMF2* in broccoli (*Brassica oleracea* var. *italica* cv. Elegance) development, we identified two broccoli *EMF2* (*BoEMF2*) genes with sequence homology to and a similar gene expression pattern to that in Arabidopsis (*AtEMF2*). Reducing their expression in broccoli resulted in aberrant phenotypes and gene expression patterns. *BoEMF2* regulates genes involved in diverse developmental and stress programs similar to *AtEMF2* in Arabidopsis. However, *BoEMF2* differs from *AtEMF2* in the regulation of flower organ identity, cell proliferation and elongation, and death-related genes, which may explain the distinct phenotypes. The expression of *BoEMF2.1* in the Arabidopsis *emf2* mutant (Rescued *emf2*) partially rescued the mutant phenotype and restored the gene expression pattern to that of the wild type. Many *EMF2*-mediated molecular and developmental functions are conserved in broccoli and Arabidopsis. Furthermore, the restored gene expression pattern in Rescued *emf2* provides insights into the molecular basis of PcG-mediated growth and development.**

**Keywords:** *Brassica oleracea* var. *italica* • Broccoli • *EMBRYONIC FLOWER 2* • Flowering • Polycomb Group Protein.

**Abbreviations:** AG, AGAMOUS; AP3, APETALA3; *asBoEMF2.1*, broccoli harboring 35S::antisense*BoEMF2.1*; CDS, coding sequence; CLF, CURLY LEAF; *COR15A*, COLD REGULATED 15A; *EMF2*, *EMBRYONIC FLOWER 2*; *EXPA*, *EXPASIN A*; FIE, FERTILIZATION INDEPENDENT ENDOSPERM; FIS2, FERTILIZATION INDEPENDENT SEED 2; *FLC*, FLOWERING LOCUS C; *FT*, FLOWERING LOCUS T; *LTP*, LIPID TRANSFER PROTEIN; MEA, MEDEA; MS, Murahige and Skoog; MSI1, MULTICOPY SUPPRESSOR OF IRA1; PcG, polycomb group; *PI*, *PISTILLATA*; PRC, polycomb repressive

complex; qRT-PCR, quantitative real-time reverse transcription-PCR; RACE, rapid amplification of cDNA ends; Rescued *emf2*, *emf2* mutant harboring 35S::*BoEMF2.1*; RFLP, restriction fragment length polymorphism; *SEP3*, *SEPATA*3; Su[z]12, Suppressor of Zeste 12; *SVP*, *SHORT VEGETATIVE PHASE*; *SWN*, *SWINGER*; *TFL2*, *TERMINAL FLOWER 2*; transWT, 35S::*BoEMF2.1* containing WT; *VRN2*, *VERNALIZATION2*; WT, wild type.

The nucleotide sequences reported in this paper has been submitted to GenBank under the accession numbers JQ412719 (*BoEMF2.1* cDNA), JQ412720 (*BoEMF2.2* cDNA), JQ412722 (*BoEMF2.1* genomic DNA) and JQ412723 (*BoEMF2.2* genomic DNA). The Arabidopsis and broccoli microarray data were submitted to Gene Expression Omnibus (GEO) database under the accession numbers GSE36943 and GSE36963, respectively.

## Introduction

Polycomb group (PcG) proteins are epigenetic repressors in eukaryotes known to maintain the silent states of their target genes (Morey and Helin 2010, Margueron and Reinberg 2011). PcG proteins form multiprotein complexes, such as polycomb repressive complex 1 and 2 (PRC1 and PRC2), which modify histone moieties and remodel chromatin structures to repress transcriptional activities (Schuettengruber et al. 2007). PRC2 has histone methyl transferase activity that trimethylates histone 3 lysine 27 (H3K27) on target genes, whereas PRC1 catalyzes histone 2A (H2A) monoubiquitination (Muller and Verrijzer 2009). Together, these PcGs confer stable gene silencing (Morey and Helin 2010, Margueron and Reinberg 2011).

PRC2, first described in *Drosophila*, is composed of four core proteins (Muller et al. 2002). In plants, Arabidopsis proteins homologous to components of *Drosophila* PRC2 are required for three developmental functions (Calonje and Sung 2006, Hennig and Derkacheva 2009, Holec and Berger 2012). Arabidopsis FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) are

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homologs to *Drosophila* Extra sex comb (ESC) and p55, respectively. CURLY LEAF (CLF), MEDEA (MEA) and SWINGER (SWN) are homologous of Enhancer of Zeste (E[z]), which catalyzes the trimethylation of H3K27 on target genes. EMBRYONIC FLOWER 2 (EMF2), VERNALIZATION2 (VRN2) and FERTILIZATION INDEPENDENT SEED2 (FIS2) are homologous to Suppressor of Zeste 12 (Su[z]12) (Calonje and Sung 2006, Hennig and Derkacheva 2009, Holec and Berger 2012). The three Arabidopsis homologous proteins VRN2, EMF2 and FIS2 share several amino acid sequence domains with their *Drosophila* Su[z]12 homologs, including the VEFs domain (Yoshida et al. 2001). These three proteins also play a major role in determining the biological function of the three PRC2 complexes by regulating specific target genes. EMF2, together with MSI1, FIE and CLF or SWN (EMF2/CLF or SWN/FIE/MSI1), maintains vegetative growth by repressing the expression of the flower MADS-box genes AGAMOUS (AG), APETALA 3 (AP3) and PISTILLATA (PI) (Moon et al. 2003, Calonje et al. 2008). FIS2/MEA/FIE/MSI1 regulates Arabidopsis seed development by repressing PHERES1 during gametophyte and endosperm development (Kohler et al. 2003). Likewise, VRN2/CLF or SWN/FIE/MSI1 is involved in vernalization-mediated flowering by regulating FLOWERING LOCUS C (FLC) (Sung and Amasino 2004, Wood et al. 2006, De Lucia et al. 2008).

Similarly, Arabidopsis proteins homologous to *Drosophila* PRC1 components such as the RING-finger homologs AtRING1A/B and AtBMI1A/B are required for maintaining cell identity in Arabidopsis (Sanchez-Pulido et al. 2008, Xu and Shen 2008, Bratzel et al. 2010). These proteins interact with LIKE HETEROCHROMATIN PROTEIN1 (LHP1)/TERMINAL FLOWER2 (TFL2) and EMF1, and catalyze histone 2A (H2A) monoubiquitination (Bratzel et al. 2010, Chen et al. 2010).

EMF2-like sequences exist in many plants (Chen et al. 2009, Luo et al. 2009); however, their function has been studied only in the model plants Arabidopsis (Luo et al. 1999, Gendall et al. 2001, Yoshida et al. 2001) and rice (Luo et al. 2009). Mutations in Arabidopsis and rice EMF2 cause early flowering and affect flower organ development (Yoshida et al. 2001, Luo et al. 2009). However, the *emf2* mutant is pleiotropic (Yoshida et al. 2001) and the mutation affects the expression of genes in various genetic programs, including leaf, stem and flower development; hormone synthesis and response; and stress responses (Moon et al. 2003, Kim et al. 2010).

To investigate the function of EMF2 in other plant species, we studied the close relative of Arabidopsis, broccoli (*Brassica oleracea* var. *italica* cv. Elegance) from *Brassicaceae*. Broccoli is a paleopolyploid (Qiu et al. 2009) that diverged from a common ancestor with Arabidopsis about 14.5–20.4 million years ago (Yang et al. 1999) but differs in gross morphological features from Arabidopsis. To study the function of broccoli EMF2 (BoEMF2), we cloned two AtEMF2 homologs, BoEMF2.1 and BoEMF2.2. Both BoEMF2 knockdown in broccoli and expression of BoEMF2 in Arabidopsis *emf2* mutants showed that BoEMF2s are similar to the Arabidopsis homolog in being required for broccoli growth and differentiation. Unlike

AtEMF2, BoEMF2s do not seem to promote cell elongation and are needed to prevent wilting and premature death. We discuss the role of PcG gene modulation in morphological differentiation during evolution.

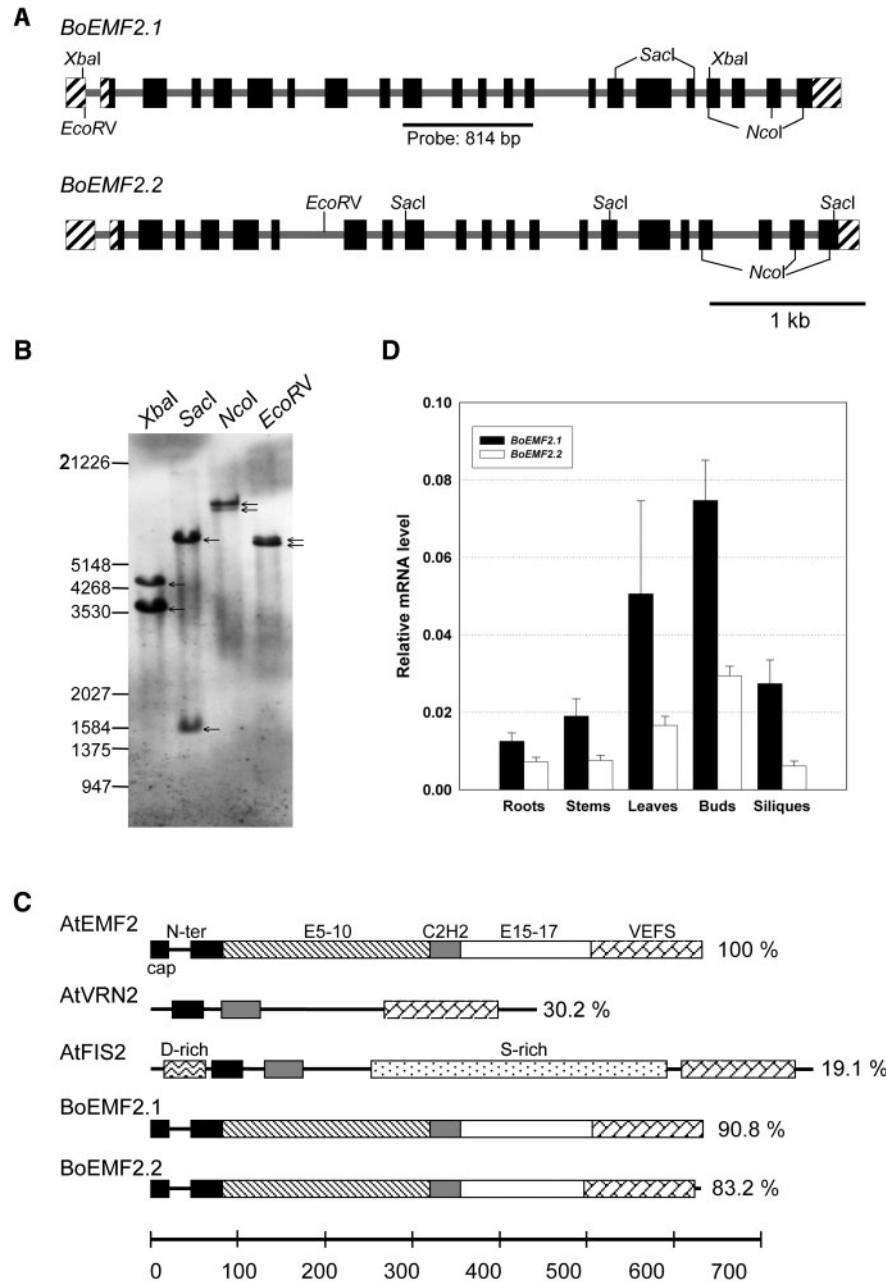
## Results

### Two BoEMF2 genes identified and characterized in broccoli

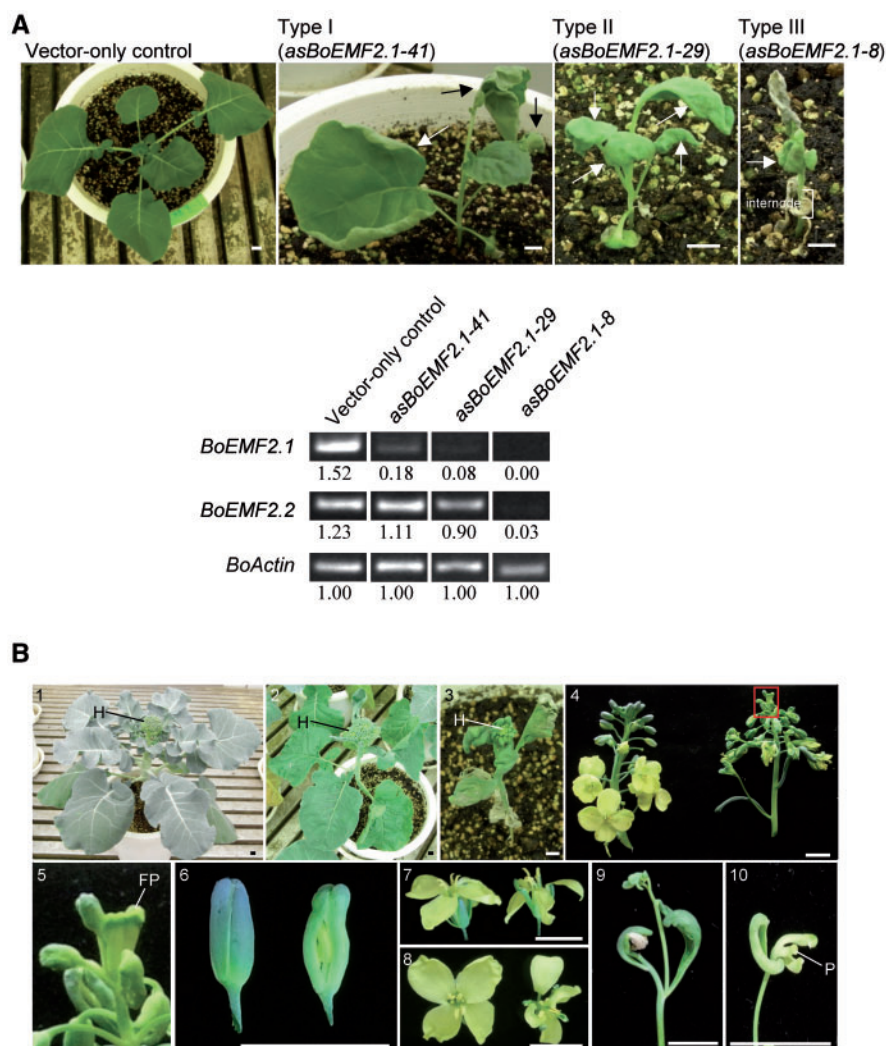
Two broccoli (*B. oleracea* var. *italica*) EMF2 (BoEMF2) genes, BoEMF2.1 and BoEMF2.2, were cloned by combining chromosome walking and rapid amplification of cDNA ends (RACE) with primers based on Arabidopsis sequences (Supplementary Fig. S1, Supplementary Table S1). BoEMF2.1 and BoEMF2.2 are both composed of 22 exons (Fig. 1A). Their coding regions are 1,896 and 1,890 nucleotides, and show 90.6 and 86% similarity, respectively, to Arabidopsis EMF2 (AtEMF2). Southern blot analysis with a probe specific to BoEMF2.1 exon 10–14 (Fig. 1A) that showed 81% similarity to that of BoEMF2.2 confirmed the cloning of two copies of BoEMF2s in the broccoli genome (Fig. 1B). *Xba*I, *Sac*I, *Nco*I and *Eco*RV were chosen to digest broccoli genomic DNA. There are two *Xba*I, two *Sac*I, three *Nco*I and one *Eco*RV cutting site on the BoEMF2.1 genome whereas there are three *Sac*I, three *Nco*I and one *Eco*RV cutting site on the BoEMF2.2 genome. As shown in Fig. 1B, digestions of broccoli genomic DNA with *Xba*I, *Sac*I, *Nco*I and *Eco*RV all produced one strong and one weak band. The strong band represented the DNA fragment of BoEMF2.1 and the weak one represented that of BoEMF2.2 (Fig. 1B) due to the fact that the probe is 100% match to the BoEMF2.1 sequence.

Protein sequences of BoEMF2.1 and BoEMF2.2 show 82.2% identity to each other, and 90.8 and 83.2%, respectively, to AtEMF2. In addition to their high protein identity, BoEMF2s and AtEMF2 share a common domain organization (Chen et al. 2009): an N-terminal cap, an N-terminal domain, E5–10 (exon 5–exon10), a C2H2 domain, E15–17 (exon 15–exon 17) and a C-terminal acidic-W/M (VEFS) domain (Fig. 1C). The VEFs domains for BoEMF2.1 and BoEMF2.2 share 87.4% identity to each other, and 93.7 and 88.2%, respectively, to AtEMF2. The two BoEMF2 proteins are quite different from the other two Arabidopsis Su[z]12 homologs, AtVRN2 and AtFIS2 (Chen et al. 2009). AtVRN2 and AtFIS2 show only 30.2 and 19.1% protein identity to AtEMF2, respectively. Of the protein domains, both AtVRN2 and AtFIS2 lost the N-terminal cap region, and the E5–10 and E15–17 domains present in AtEMF2 and the BoEMF2s. In addition AtFIS2 protein acquired a specific aspartic acid-rich (D-rich) domain at the N-terminus and a serine-rich (S-rich) domain flanking the C2H2 and VEFs domains (Fig. 1C).

Real-time quantitative reverse transcription–PCR (qRT–PCR) of the expression of the BoEMF2 genes with *Brassica Actin* (BoActin) used as a control revealed ubiquitous expression in all organs studied (Fig. 1D). Both genes were most highly expressed in flower buds relative to roots, leaves, stems and



**Fig. 1** Characterization of *BoEMF2.1* and *BoEMF2.2*. (A) Gene structure of the two *BoEMF2* genes. The box represents the exon and the connecting gray line represents the intron of the two genes. The 5'- and 3'-non-coding regions are shown as boxes with diagonal lines. The restriction sites of *Xba*I, *Sac*I, *Nco*I and *Eco*RV, and the region for probe synthesis for Southern blot analysis are indicated. The scale bar represents 1 kbp of nucleotides. (B) Southern blot analysis. Genomic DNA was digested with use of *Xba*I, *Sac*I, *Nco*I and *Eco*RV, and detected with a *BoEMF2.1*-specific digoxigenin labeling probe on a PVDF membrane. Arrows indicate the enzyme-digested DNA fragments. The size of the marker in base pairs is indicated at the left. (C) Protein identity and domain organization. The N-terminal domain (N-ter) is composed of two parts including an N-terminal cap (cap), E5-10, C2H2 zinc finger, E15-17 and VEFS domain conserved in At- and BoEMF2s. The D-rich and S-rich domain are specific to AtFIS2. Protein identity is indicated at the right. The scale bar represents the length of the protein in amino acids. (D) Tissue-specific expression pattern. The y-axis indicates the relative mRNA level normalized to *BoActin*. Data are the mean with SD from three independent experiments.



**Fig. 2** Phenotypes and expression of *BoEMF2* genes in *asBoEMF2.1* transgenic plants. (A) Six-week-old vector-only control and three types of *asBoEMF2.1* transgenic broccoli are shown in the upper panel: type I *asBoEMF2.1-41*, type II *asBoEMF2.1-29* and type III *asBoEMF2.1-8*. The lower panel shows the mRNA level of *BoActin* and *BoEMF2* genes in control and transgenic plants. PCR cycles for *BoEMF2.1*, *BoEMF2.2* and *BoActin* were 30, 30 and 24, respectively. The Arabic numbers indicate the expression of genes relative to that of *BoActin*, used as a control. The arrows indicated the curled leaves. (B) Adult plants at the inflorescence stage of control (1), type I *asBoEMF2.1* (2) and type II *asBoEMF2.1* (3). Elongated inflorescence (4), flower bud (6), open flower (7 and 8) of control (left) and type I *asBoEMF2.1* (right). An enlarged view of the red box in 2B-4, showing a fused pistil-like structure (FP) on the apex of a type I *asBoEMF2.1* inflorescence (5). Inflorescence (9) and abnormal flower (10) of type II *asBoEMF2.1*. H, broccoli head. P, pistil. Bars represent 1 cm.

siliques. The expression pattern for both is similar to that of *AtEMF2*.

Thus, *BoEMF2* genes are similar to their Arabidopsis homolog in terms of sequence, domain organization and gene expression pattern. We knocked down the expression of the *BoEMF2* genes in broccoli to study their biological function and introduced *BoEMF2.1* into Arabidopsis *emf2* mutants to determine whether *BoEMF2.1* can rescue the *AtEMF2* function.

### Reducing *BoEMF2* activity affected vegetative and flower development in broccoli

To investigate the role of *BoEMF2* in broccoli growth and development, we used reverse genetics to knock down the

expression of the *BoEMF2* genes by introducing the full-length coding sequence (CDS) of antisense *BoEMF2.1* under the control of a constitutive *Cauliflower mosaic virus* promoter 35S (35S::*antisenseBoEMF2.1*) into broccoli. Compared with plants transformed with the empty vector (vector-only control), T<sub>1</sub> and T<sub>2</sub> generations of transgenic broccoli harboring 35S::*antisenseBoEMF2.1* (*asBoEMF2.1*) showed aberrant phenotypes during development (Fig. 2; Supplementary Fig. S2). For the T<sub>1</sub> plants, 6 weeks after germination, leaves tended to curl upwards. The extent of curl was inversely related to the level of endogenous *BoEMF2* genes (Fig. 2; Supplementary Fig. S2). We could classify three types of *asBoEMF2.1* transgenic plants by their morphological features.

Type I plants showed a few mildly curly leaves and expression of *BoEMF2.1* reduced as compared with the wild type (WT) level (*asBoEMF2.1-41* in **Fig. 2A**; *asBoEMF2.1-1, -7, -10, -16, -23, -25, -39* and *-41* in **Supplementary Fig. S2**), whereas the RNA level of *BoEMF2.2* in these plants was not as affected as in type I plants. Type II *asBoEMF2.1* plants were dwarf, had tightly curled leaves and showed almost completely knocked-out expression of *BoEMF2.1* and a clear reduction in the *asBoEMF2.2* level (*asBoEMF2.1-29* in **Fig. 2A**; *asBoEMF2.1-14, -29* and *-32* in **Supplementary Fig. S2**). Type III *asBoEMF2.1* plants had a few tightly curled leaves that wilted as new leaves emerged and died prematurely within a few days in the soil. Interestingly, unlike the *emf2* mutation that greatly reduced internode and petiole elongation in Arabidopsis, type III plants appeared to have a normal petiole and internode length (*asBoEMF2.1-8* in **Fig. 2A**). These plants had non-detectable *BoEMF2.1* levels and almost completely knocked-out expression of *BoEMF2.2* (*asBoEMF2.1-8* in **Fig. 2A**; *asBoEMF2.1-5* and *-8* in **Supplementary Fig. S2**).

The vector-only control, type I and type II *asBoEMF2.1* plants could undergo reproductive growth and produce flowers. The *asBoEMF2.1* broccoli flowered earlier than control plants (**Table 1**):  $69.0 \pm 5.4$  d after germination and producing  $12.8 \pm 1.4$  leaves as compared with  $87.7 \pm 5.2$  d with  $16.2 \pm 1.0$  leaves for controls. Compared with control plants (**Fig. 2B-1**), *asBoEMF2.1* type I (**Fig. 2B-2**) and type II (**Fig. 2B-3**) plants produced thinner stems, fewer leaves, abnormal flowers and, sometimes, a determinate inflorescence (**Fig. 2B-4, -5**). These features resulted in an abnormal inflorescence profile in *asBoEMF2.1* plants (**Fig. 2B-4**).

Type I *asBoEMF2.1* plants produced many flowers, thus forming a small broccoli head (**Fig. 2B-2**). The sepals are not fully enclosed to protect the inner flower organs in the flower bud (**Fig. 2B-6**). In the open flowers, the petals and sepals were irregular in shape and smaller (**Fig. 2B-7, -8**). Occasionally, fused pistil-like structures were observed on the top of the inflorescence, which suggests determinate growth (**Fig. 2B-4, -5**). Few seeds could be collected from type I plants. Type II *asBoEMF2.1* plants produced many to a few flower buds (**Fig. 2B-9**) with imperfect flowers, usually lacking petals and stamens (**Fig. 2B-10**), with barely any seed produced.

In summary, *asBoEMF2.1* showed knocked-down endogenous *BoEMF2.1* and *BoEMF2.2* expression, which is consistent with the sequence similarity of the two *BoEMF2* genes.

**Table 1** Flowering time in *asBoEMF2.1* and vector-only control transgenic broccoli

| Transgenic plants <sup>a</sup> | Days after germination | Number of leaves |
|--------------------------------|------------------------|------------------|
| Vector-only control            | $87.7 \pm 5.23$        | $16.2 \pm 1.03$  |
| <i>asBoEMF2.1</i> <sup>b</sup> | $69.0 \pm 5.42$        | $12.8 \pm 1.42$  |

<sup>a</sup> The number of plants used for analysis was 10 vector-only control and 12 type I (9) and type II (3) *asBoEMF2.1*.

<sup>b</sup> Type I and type II *asBoEMF2.1* broccoli with knockdown expression of endogenous *BoEMF2* genes. Type I plants flowered  $67.3 \pm 5.29$  d after germination and produced  $13.0 \pm 1.58$  leaves, whereas type II plants flowered after  $74 \pm 0$  d and produced  $12.0 \pm 0$  leaves.

Knocking down both *BoEMF2* genes caused more severe phenotypes than knocking down only *BoEMF2.1*, so the two *BoEMF2* genes must have redundant functions and have a dosage effect on leaf morphologic features, vegetative growth and flower development in broccoli.

### Global gene expression pattern of *asBoEMF2.1* transgenic broccoli

To study the underlying molecular mechanism of the aberrant phenotypes caused by the reduced *BoEMF2* expression, we studied the global gene expression pattern of 11-day-old *asBoEMF2.1* and the vector-only control by microarray assay (Redman et al. 2004). The 11-day-old *asBoEMF2.1* plants with one to two true leaves were used to determine *BoEMF2* RNA levels. Individual seedlings were genotyped to identify those with knocked-down expression. Only type I and type II *asBoEMF2.1* broccoli were found but not type III plants because of their delayed germination and slow growth. In comparing the gene expression pattern for the *asBoEMF2.1* transgenic and vector-only controls, we identified 2,973 genes up-regulated and 3,369 genes down-regulated in *asBoEMF2.1*. Similar to the Arabidopsis *emf2* mutant, *asBoEMF2.1* broccoli showed ectopic expression of genes in most of the 15 functional categories described by Kim et al. (2010) (**Table 2**; **Supplementary Table S2**). About 16% of genes ectopically expressed in *emf2* were also ectopically expressed in *asBoEMF2.1* broccoli, including the flower organ identity genes *AG* and *SEPATALLA3* (*SEP3*), the flowering time gene *FLOWERING LOCUS T* (*FT*), the seed maturation *LIPID TRANSFER PROTEIN* family genes (*LTP*) and late embryo abundant protein genes (*LEA*), genes encoding transcription factors and genes involved in hormone and stress responses (**Table 2**). The ectopic expression of these flower organ identity genes is consistent with the abnormal flower development and early flowering of *asBoEMF2.1* broccoli (**Fig. 2**).

To confirm the microarray results, we used RT-PCR to examine the expression of broccoli *SEP3* (*BoSEP3*), *BoAG* and *BoPI*. The Affymetrix ATH1 chip experiment showed *BoSEP3* and *BoAG* up-regulation, but not *BoPI*, in *asBoEMF2.1* (**Supplementary Table S2**). RNA was extracted from two independent 11-day-old *asBoEMF2.1* transgenic plants (*G*<sub>1</sub> and *G*<sub>2</sub>), the 11-day-old vector-only control (*C*), and flower (*Cf*) and silique (*Csi*) from a vector-only control plant. The *BoEMF2* mRNA levels were reduced in the two *asBoEMF2.1* broccoli seedlings. The *G*<sub>1</sub> seedling showed a reduced *BoEMF2* mRNA level relative to the control: 18% for *BoEMF2.1* and 48% for *BoEMF2.2*, and the *G*<sub>2</sub> seedling showed complete knockout of *BoEMF2.1* and 35% *BoEMF2.2* expression as compared with the vector-only control (**Fig. 3**). In the control broccoli, *BoSEP3* and *BoAG* were preferentially expressed in flower (*Cf*) and silique (*Csi*), whereas *BoPI* expression was specific to flowers. In the 11-day-old *asBoEMF2.1*, both *BoSEP3* and *BoAG* were up-regulated but not *BoPI*, which was repressed, as in control seedlings (**Fig. 3**). The RT-PCR results for flower organ identity genes were consistent with the microarray results and suggest that *BoEMF2.1* and *AtEMF2* regulate similar, but not identical, target genes.

**Table 2** Categories of genes up-regulated in both *emf2* and *asBoEMF2.1*

| Category              | Genes up-regulated by >2-fold in <i>asBoEMF2.1</i> broccoli <sup>a</sup>   |
|-----------------------|--|
| Flower organ identity | <i>AG</i> <sup>b,c</sup> , <i>AP3</i> <sup>b,c,d</sup> , <i>BEL1</i> <sup>c</sup> , <i>SEP3</i> <sup>b,c</sup>   |
| Flowering time        | <i>ATC</i> <sup>c</sup> , <i>FT</i> <sup>b,c,d</sup>   |
| Seed                  | <i>LEA</i> <sup>b,c</sup> , <i>LTP3</i> <sup>b,c</sup> , <i>LTP4</i> <sup>b,c</sup> , <i>SAG21</i> <sup>b</sup> ,<br>Protease inhibitor genes <sup>b,c</sup>   |
| Gibberellin           | <i>RGL1</i> <sup>b</sup>   |
| Ethylene              | <i>ERF12</i> <sup>b</sup> , <i>MBF1C</i> <sup>c</sup>  |
| ABA                   | <i>HVA22D</i> <sup>b,c</sup> , <i>OST1</i> <sup>c</sup>  |
| Cold                  | <i>CBF1</i> <sup>c</sup> , <i>DREB1A</i> <sup>c</sup>  |
| Stress                | <i>KIN1</i> <sup>b,c</sup> , <i>STZ</i> <sup>c</sup>   |
| Heat                  | <i>HSF4</i> <sup>b,c</sup> , <i>HSFA2</i> <sup>c</sup> , <i>HSP17.6</i> <sup>b,c</sup> , <i>HSP101</i> <sup>b,c</sup>  |
| Photoreceptor         | <i>CSN6A</i> <sup>c</sup> , <i>FUS12</i> <sup>c</sup>  |
| Expansin              | <i>EXPA7</i> <sup>b,c</sup>  |
| Transcription factor  | <i>AGL57</i> <sup>b</sup> , <i>AF1</i> <sup>b,c</sup> , <i>ANAC087</i> <sup>b,c</sup> ,<br><i>COL9</i> <sup>b</sup> , <i>MYB96</i> <sup>c</sup> , <i>NAC1</i> <sup>b</sup> , <i>NAM</i> <sup>b,c</sup> |

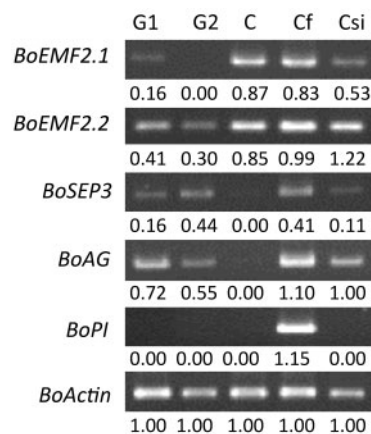
<sup>a</sup> See **Supplementary Table S2** for additional up-regulated genes.<sup>b</sup> Genes up-regulated in *emf2* based on data in the current study.<sup>c</sup> Gene up-regulated in *emf2* based on data in Kim *et al.* (2010).<sup>d</sup> Genes up-regulated only 1.9-fold in *asBoEMF2.1* broccoli.

*AF1*, NAC DOMAIN CONTAINING PROTEIN 2; *AGL57*, AGAMOUS-LIKE 57; *ATC*, ARABIDOPSIS THALIANA CENTRORADIALIS; *ANAC087*, NAC DOMAIN CONTAINING PROTEIN 87; *BEL1*, BELL 1; *CBF1*, C-REPEAT/DRE BINDING FACTOR 1; *COL*, CONSTANS-LIKE; *CSN6A*, COP9 SIGNALOSOME COMPLEX SUBUNIT 6A; *DREB1A*, DEHYDRATION RESPONSE ELEMENT B1A; *EXPA*, EXPANSIN A; *FUS12*, FUSCA 12; *HSF1*, HEAT SHOCK TRANSCRIPTION FACTOR C1; *HSP*, HEAT SHOCK PROTEIN; *HVA22D*, HVA22 HOMOLOGUE D; *NAC1*, NAC DOMAIN CONTAINING PROTEIN 1; *NAM*, NO APICAL MERISTEM; *NIK3*, NSP-INTERACTING KINASE 3; *MBF1C*, MULTIPROTEIN BRIDGING FACTOR 1C; *MYB96*, MYB DOMAIN PROTEIN 96; *OST1*, OPEN STOMATA 1; *RGL1*, RGA-LIKE 1; *SAG21*, SENESCENCE-ASSOCIATED GENE 21; *STZ*, SALT TOLERANCE ZINC FINGER.

### **BoEMF2.1 partially rescued the Arabidopsis *emf2* phenotype**

The two *BoEMF2* genes are similar to *AtEMF2* in sequence, protein domain organization and expression pattern. Because the expression of *BoEMF2.1* was higher than that of *BoEMF2.2* in broccoli and the former showed higher sequence similarity to *AtEMF2* than *BoEMF2.2*, we used *BoEMF2.1* to investigate whether it can substitute for the *AtEMF2* function. We generated a 35S::*BoEMF2.1* full-length CDS construct and introduced it into Arabidopsis *emf2* mutants to determine whether *BoEMF2.1* can restore normal seedling and adult development in *emf2*. Hygromycin-resistant transgenic plants harboring the *emf2* mutation were identified by restriction fragment length polymorphism (RFLP) genotyping (**Supplementary Fig. S3A**) as described (Calonje *et al.* 2008).

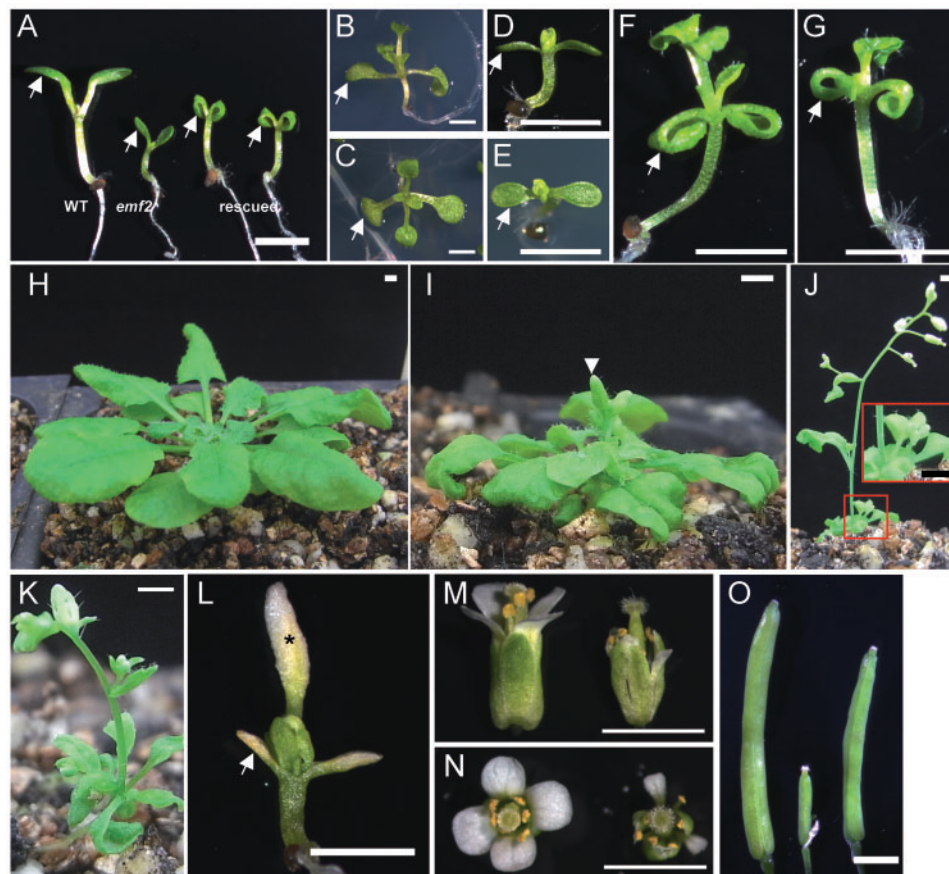
Analysis of the phenotypes of the homozygous *emf2* mutants harboring 35S::*BoEMF2.1* (Rescued *emf2*) grown under short-day conditions showed that *BoEMF2.1* partially rescued *emf2* phenotypes (**Fig. 4, Table 3**).



**Fig. 3** RT-PCR analysis of *BoEMF2* genes and flower MADS-box gene expression in *asBoEMF2.1* and control broccoli. mRNA level of the genes in 11-day-old seedlings. G<sub>1</sub> and G<sub>2</sub>, two independent *asBoEMF2.1* transgenic broccoli; C, a vector-only control line; Cf, flower of the vector-only control; Csi, silique of the control line. The mRNA level was normalized to that of *BoActin* and is presented as Arabic numbers beneath each gel photo. The unsaturated PCR cycles for *BoEMF2.1*, *BoEMF2.2*, *BoSEP3*, *BoAG*, *BoPI* and *BoActin* were 33, 33, 27, 30, 24 and 30, respectively.

At 7 d after germination, *emf2* mutants showed short hypocotyls, petioleless, and small and oval-shaped cotyledons (Chen *et al.* 1997). *BoEMF2.1* partially rescued the hypocotyl and petiole elongation in *emf2* mutants (**Fig. 4A, Table 3**). The petioleless cotyledons of *emf2* were slightly curled upwards, whereas the cotyledons of Rescued *emf2* were curled downwards along the proximodistal axis, and the WT cotyledons were flat and round (**Fig. 4A**).

At 15 d, WT seedlings produced four petiolated rosette leaves and the cotyledons continued to grow (**Fig. 4B, C**), whereas the cotyledons of *emf2* stopped growing, and no petiolated rosette leaves were produced (**Fig. 4D, E**). Rescued *emf2* produced petiolated rosette leaves to varying extents (**Fig. 4F, G**). The hypocotyl was longer for Rescued *emf2* than *emf2* but shorter than for the WT (**Fig. 4A–G, Table 3**). The 15-day-old WT and Rescued *emf2* were transplanted into the soil for further observation, and *emf2* seedlings remained on agar plates because they cannot survive in soil. The Rescued *emf2* produced about six petiolated rosette leaves, which were smaller than those of the WT (**Fig. 4H–K**) before flowering at about 30 d after germination; *emf2* mutants flowered at about 22 d and produced no rosette leaves, and the WT flowered at about 104 d and produced >64 rosette leaves (**Table 3**). Therefore, *BoEMF2.1* partially rescued vegetative growth in *emf2*, although the rescued plants were smaller and flowered early. The Rescued *emf2* produced a normal, although smaller inflorescence than the WT (**Fig. 4J, K**). *emf2* plants produced terminal flowers with a large pistil and dwarfed short stamens (**Fig. 4L**). Rescued *emf2* showed indeterminate growth and secondary inflorescences developed from the axillary meristems (**Fig. 4J, K**), like WT plants.



**Fig. 4** Partial rescue of *Arabidopsis emf2* by *BoEMF2.1*. (A) Seven-day-old WT (left), *emf2* (middle) and Rescued *emf2* (right). (B and C) Fifteen-day-old WT. (D and E) Fifteen-day-old *emf2*. (F and G) Fifteen-day-old Rescued *emf2*. (H) Six-week-old WT at the vegetative stage. (I–K) Six-week-old Rescued *emf2* at the reproductive stage with varying degrees of rescue in leaf size and vegetative development. The arrowhead indicates the emerging inflorescence. The red box in J shows an enlarged view of a petiolated rosette leaf. (L) Six-week-old *emf2* produced a pistil directly from the embryonic shoot apical meristem (\*). (M) A lateral view of the WT flower (left) and Rescued *emf2* flower (right) showing the smaller petals and shorter stamens. (N) Aerial view of the WT flower (left) and Rescued *emf2* flower (right). (O) WT silique (left), with a spontaneously developed silique, a short and empty silique in the Rescued *emf2* without manual pollination (middle), and a silique of Rescued *emf2* after manual self-pollination (right). The arrow indicates the cotyledon. The bar represents 2 mm.

**Table 3** Hypocotyl length of 15-day-old *Arabidopsis* seedlings and leaf number at bolting

| Genotype            | Hypocotyl length <sup>a</sup> (mm, <i>n</i> = 20) | Bolting time <sup>b</sup> (day, <i>n</i> = 20) | Rosette leaf number at bolting ( <i>n</i> = 20) |
|---------------------|---|--|---|
| Wild type           | 7.0 ± 0.5   | 104.4 ± 5.4                                    | 64.5 ± 2.2                                      |
| <i>emf2</i>         | 3.1 ± 0.3   | 22.9 ± 2.9                                     | 0   |
| Rescued <i>emf2</i> | 5.3 ± 0.7   | 30.6 ± 2.8                                     | 5.9 ± 2.5                                       |

<sup>a</sup> Fifteen-day-old seedlings grown under short-day conditions on agar plates.

<sup>b</sup> *Arabidopsis* transferred from agar at 14 d to soil and grown under short-day conditions until bolting.

Flower development in *emf2* was partially rescued by the constitutive expression of *BoEMF2.1*. Rescued *emf2* produced flower organs of relatively normal size: unlike petal-less and extremely dwarfed stamens in *emf2*, petals were present, although smaller; the pistil was only slightly taller than stamens

(Fig. 4M, N). However, partially rescued flowers did not produce seeds and the siliques did not elongate spontaneously (Fig. 4O), probably because of the slightly shorter filaments, smaller anthers and fewer pollen produced than the WT. Therefore, we performed manual pollination by placing the entire anther from the same flower or the same plant onto the pistil and generated many seeds in siliques of relatively normal size (Fig. 4O), but still fewer than the WT.

Thus, broccoli *BoEMF2.1* can substitute for *Arabidopsis AtEMF2* in performing developmental functions in part at every stage of *Arabidopsis* development.

### **BoEMF2.1 partially restored the expression of misexpressed genes in the *emf2* mutant**

To understand the molecular basis of the partial gene rescue, we studied the global gene expression pattern of Rescued *emf2* seedlings. The genotype and expression of *BoEMF2.1* were

characterized to ensure that the Rescued *emf2* plants selected based on morphological features were indeed the *emf2* mutants harboring *BoEMF2.1* (Supplementary Fig. S3A, B). RNA was extracted from 7-day-old Rescued *emf2*, *emf2*, WT, and WT-like transgenic seedlings harboring *BoEMF2.1* (transWT) for microarray analysis as described in the Materials and Methods.

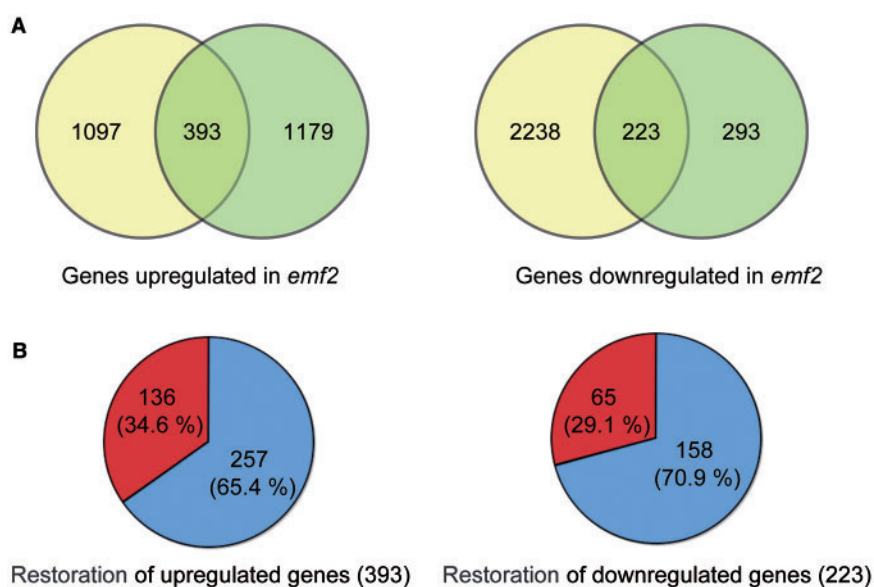
We first examined the *emf2* expression pattern using the 22 K Affymetrix ATH1 microarray and found 1,490 and 2,461 genes up- and down-regulated, respectively, by >2-fold in *emf2*. About 94% of the genes misregulated in the current study were detected on the custom GeneChip reported by Kim et al. (2010). The two independent data sets showed an overlap of 393 and 223 genes up- and down-regulated, respectively, in *emf2* seedlings (Fig. 5A; Supplementary Table S3), which represent a robust set of EMF2-regulated genes. As reported by Kim et al. (2010), we found misexpression of genes in *emf2* in the 15 functional categories (Supplementary Tables S3, S4), including flower organ identity genes *PI*, *AP3*, *SEP3* and *AG*; seed maturation genes *LTP3* and *LTP4*; cell growth genes *EXPANSIN A* (*EXPA*); flowering time genes *FT*, *FLC*, *SHORT VEGETATIVE PHASE* (*SVP*); a COLD REGULATED 15A (*COR15A*) gene; and the heat-shock protein (*HSP*) *HSP 70* gene.

We then studied the global gene expression pattern in the Rescued *emf2*. The control used was transWT, which is a transgenic plant harboring *BoEMF2.1* in the WT background and phenotypically indistinguishable from the WT, and hence a better control than the WT, which does not harbor any transgene. A comparison of the gene expression patterns of Rescued *emf2* normalized to transWT and *emf2* normalized to WT revealed 1,122 of the 1,490 genes up-regulated in *emf2* (75%) no longer up-regulated and 1,899 of the 2,461 down-regulated

genes in *emf2* (77%) no longer down-regulated. Of the robust set of EMF2-regulated genes, 257 of the 393 genes up-regulated in *emf2* (65%) were no longer up-regulated (Fig. 5B) and 158 of the 223 down-regulated genes in *emf2* (71%) were no longer down-regulated (Fig. 5B), which suggests that 35S::*BoEMF2.1* restored the misregulation of many genes in *emf2*. Further analysis showed that the expression of genes misregulated in all functional categories was restored, for many, to near-normal levels (Table 4; Supplementary Table S4).

The reduced expression of flower organ identity genes *AP1*, *AP3* and *PI*, and changed expression of flowering time genes *FT* and *SVP* are consistent with the normal flower organ development and delayed flowering, respectively, in *emf2* harboring *BoEMF2.1*. *BoEMF2.1* also restored the expression of the gibberellic acid synthesis gene *GIBBERELLIN 2-OXIDASE 2* (*GA2OX2*), stress-related genes *C-REPEAT/DRE BINDING FACTOR 1* (*CBF1*) and *COR15A*, and transcription factors *TEOSINTE BRANCHED1*, *CYCLOIDEA AND PCF TRANSCRIPTION FACTOR* (*TCP*), *NO APICAL MERISTEM* (*NAM*) and *CUP SHAPED COTYLEDON* (*CUC*). Many of these genes are direct targets of EMF1 and their expression is repressed by EMF1 and PRC2 (Kim et al. 2010, Kim et al. 2012). A change in the expression pattern of EMF-interacting Arabidopsis genes with *BoEMF2.1* suggests the ability of broccoli *BoEMF2.1* to act as a member of Arabidopsis PRC2 in silencing Arabidopsis genes.

Despite a high percentage of genes with expression restored by *BoEMF2.1* in *emf2*, several flower *MADS-box* genes, *AG*, *PI* and *SEP3*, were still up-regulated in Rescued *emf2* seedlings (Supplementary Table S4). To verify the microarray results, we performed qRT-PCR analysis of select genes in transWT, WT, *emf2* and Rescued *emf2*. We confirmed the increased



**Fig. 5** *BoEMF2.1* restoration of genes misexpressed in *emf2*. (A) Venn diagrams of genes up-regulated (left) and down-regulated (right) by >2-fold in *emf2* mutants in the current study (yellow) and in Kim et al. (2010) (green). (B) Pie charts of restoration of *emf2* misexpressed genes by the expression of *BoEMF2.1* in *emf2* mutants. The number (ratio) of genes with expression restored (blue) and not restored (red).

**Table 4** Restoration of expression of up- and down-regulated genes in 7-day-old *emf2* by BoEMF2.1

| Category              | No. of genes investigated | Genes misexpressed in <i>emf2</i> | No. of genes misexpressed <sup>a</sup> | No. of genes with restored expression in Rescued <i>emf2</i> <sup>b</sup> | Example of genes with expression restored to near-normal level                                  |
|-----------------------|---------------------------|-----------------------------------|--|---|---|
| Flower organ identity | 22                        | Up                                | 13                                     | 11  | <i>PI, SEP1, AP3, SHP1, SEP2, CRC, STK, AP1, SHP2, MBP2, SPL4, CRC</i>                          |
|                       |                           | Down                              | 0                                      | 0   |   |
| Flowering time        | 20                        | Up                                | 4                                      | 3   | <i>FT, ELF4</i>   |
|                       |                           | Down                              | 5                                      | 4   | <i>SVP, AGL31, RAV1</i>   |
| Seed                  | 108                       | Up                                | 35                                     | 30  | <i>LTP</i> gene family, <i>LEA, OLEO2, AIR1, SAG21, 2S</i> seed storage gene, <i>CRU2, CRU3</i> |
|                       |                           | Down                              | 19                                     | 17  | <i>NIK3, OLEO4</i>  |
| Auxin                 | 99                        | Up                                | 4                                      | 3   | Auxin-responsive protein genes  |
|                       |                           | Down                              | 21                                     | 21  | <i>DFL2, IAA2, IAA30, PIN3, PIN4, PIN7</i>  |
| Gibberellin           | 13                        | Up                                | 3                                      | 2   | <i>GA2OX2, RGL2</i>   |
|                       |                           | Down                              | 4                                      | 4   | GA-responsive protein genes   |
| Ethylene              | 53                        | Up                                | 7                                      | 7   | <i>ERF12</i> , ethylene-responsive genes  |
|                       |                           | Down                              | 9                                      | 9   | <i>EIL1, ERS2, EFE, ERF8</i>  |
| ABA                   | 12                        | Up                                | 2                                      | 0   |   |
|                       |                           | Down                              | 2                                      | 2   | <i>ABF1, ABF4</i>   |
| Cold                  | 19                        | Up                                | 5                                      | 4   | <i>COR15A</i>   |
|                       |                           | Down                              | 3                                      | 3   | <i>CBF1, CBF2, COR15B</i>   |
| Stress                | 46                        | Up                                | 2                                      | 2   | Zinc finger family protein genes  |
|                       |                           | Down                              | 6                                      | 6   | Dehydration responsive genes  |
| Heat                  | 47                        | Up                                | 10                                     | 9   | <i>HSP17.6, HSP18.2, HSP70, HSP101</i>  |
|                       |                           | Down                              | 4                                      | 4   | <i>DNAJ, HSFC1</i>  |
| Photosynthesis        | 49                        | Up                                | 0                                      | 0   |   |
|                       |                           | Down                              | 10                                     | 10  | PSII system genes   |
| Photoreceptor         | 28                        | Up                                | 1                                      | 1   | Leucine-rich repeat protein kinase gene   |
|                       |                           | Down                              | 3                                      | 3   | <i>MYB38/RAX2, ELIP1, PIF3</i>  |
| Histone               | 35                        | Up                                | 1                                      | 1   | <i>histone H1-3</i>   |
|                       |                           | Down                              | 2                                      | 2   | <i>histone H2A 12, histone 2B</i>   |
| Expansin              | 20                        | Up                                | 5                                      | 3   | <i>EXPA7, EXPA18, EXPA20</i>  |
|                       |                           | Down                              | 10                                     | 9   | <i>EXPA1, 3, 5, 6, 8, 10, 11, 15, EXPB3</i>   |
| Transcription factor  | 542                       | Up                                | 67                                     | 56  | <i>NAC1, AF1, CUC3, COL1, COL9, NAM</i>   |
|                       |                           | Down                              | 68                                     | 58  | TCP family genes  |

<sup>a</sup> Genes with  $\geq 2$ -fold up- or down-regulated expression in *emf2*.<sup>b</sup> Genes with up- or down-regulated expression in *emf2* with expression restored  $\geq 2$ -fold by BoEMF2.1.

ABF, ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTOR 1; AF1, NAC DOMAIN CONTAINING PROTEIN 2; AGL23, AGAMOUS-LIKE 23; CBF, C-REPEAT/DRE BINDING FACTOR; COL, CONSTANS-LIKE; COR, COLD-REGULATED; CRC, CRABS CLAW; CRU, CRUCIFERIN; CUC3, CUP SHAPED COTYLEDON3; DFL2, DWARF IN LIGHT 2; DNAJ, DNAJ HEAT SHOCK N-TERMINAL DOMAIN-CONTAINING PROTEIN; EFE, ETHYLENE-FORMING ENZYME; EIL1, ETHYLENE-INSENSITIVE3-LIKE 1; ELF4, EARLY FLOWERING 4; ELIP1, EARLY LIGHT-INDUCIBLE PROTEIN 1; ERF, ERF DOMAIN PROTEIN; ERS2, ETHYLENE RESPONSE SENSOR 2; EXPA, EXPANSIN A; EXPB, EXPANSIN B; GA2OX2, GIBBERELLIN 2-OXIDASE 2; HSFC1, HEAT SHOCK TRANSCRIPTION FACTOR C1; HSP, HEAT SHOCK PROTEIN; IAA, INDOLE-3-ACETIC ACID INDUCIBLE; MBP2, MYROSINASE-BINDING PROTEIN 2; NAC1, NAC DOMAIN CONTAINING PROTEIN 1; NAM, NO APICAL MERISTEM; NIK3, NSP-INTERACTING KINASE 3; MYB38/RAX2, MYB DOMAIN PROTEIN 38/REGULATOR OF AXILLARY MERISTEMS 2; OLEO, OLEOSIN; PIF, PHYTOCHROME INTERACTING FACTOR 3; PIN, PIN-FORMED; RAV1, RELATED TO ABI3/VP1 1; RGL2, RGA-LIKE 2; SAG21, SENESCENCE-ASSOCIATED GENE 21; SHP, SHATTERPROOF; SPL4, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 4; STK, SEEDSTICK; TCP, TEOSINTE BRANCHED1, CYCLOIDEA AND PCF TRANSCRIPTION FACTOR.

expression of flower *MADS-box* genes *AP3* and *PI* and the seed genes *LTP3* and *LTP4* in *emf2* but not *AG*, and *SEP3* restored in part with broccoli *EMF2.1* (Fig. 6). Similarly, the down-regulated cell growth genes *EXPA3* and *EXPA8* and the flowering time gene *SVP* were restored in part in *emf2* with *BoEMF2.1*.

In summary, the restoration of misregulated gene expression is associated with phenotypic rescue, which suggests that genes with restored expression in the Rescued *emf2* are at least in part responsible for the *emf* phenotypes.

## Discussion

### Two *BoEMF2* genes show functional conservation and redundancy in broccoli

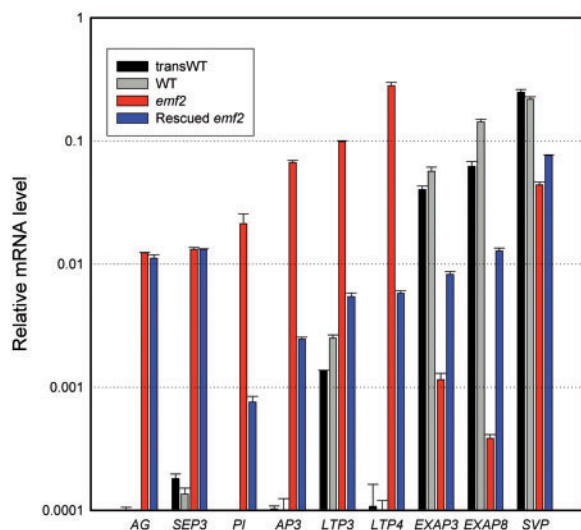
*EMF2*-like sequences common in plants (Chen et al. 2009, Luo et al. 2009) have functions distinct from their derived sequences, *VRN2* and *FIS2* (Chen et al. 2009). The two *BoEMF2* genes were identified by their high sequence identity and their similar gene expression patterns to *AtEMF2* (Fig. 1). The similar phenotypes between *emf2* mutants and *asBoEMF2.1* broccoli (Fig. 2, Table 1) and the partial rescue of *emf2* mutants by *BoEMF2.1* further confirmed their identity. The enhanced *asBoEMF2.1* phenotypes by knocking down both *BoEMF2* genes implied their functional redundancy. Reducing *EMF2* activities in Arabidopsis and broccoli caused misexpression of genes in all 15 functional categories including flower *MADS-box*, hormone signaling and *EXPA* genes, indicating the conserved molecular function of *BoEMF2* with that of *AtEMF2* in

mediating epigenetic gene silencing (Kim et al. 2010, Kim et al. 2012). Luo et al. (2009) reported a T-DNA insertional mutant of rice *EMF2* that flowered earlier than wild-type rice and produced abnormal flowers. Thus, *EMF2* is functionally conserved in regulating flowering time and organ development in Arabidopsis (Yoshida et al. 2001), rice (Luo et al. 2009) and broccoli.

### Reducing *BoEMF2* activity affected vegetative and reproductive development in broccoli

The relatively weak phenotypes displayed by type I and II *asBoEMF2.1* broccoli resemble the weak phenotypes of Arabidopsis impaired in PcG components, such as *clf* mutants (Schubert et al. 2006), *tfl2* mutants (Larsson et al. 1998, Exner et al. 2009) and Arabidopsis expressing antisense *EMF1* under the control of the *LEAFY* promoter in leaf primordia of *LFY::asEMF1* transgenic plants (Sanchez et al. 2009). Indeed, these plants can produce vegetative leaves but are smaller in size, and flower early. In addition, leaves are curly around the edges in *clf* and *LFY::asEMF1* plants. The curly leaf observed in *asBoEMF2.1* may be attributed to the ectopic expression of the flower *MADS-box* genes such as *AG*, because the curly leaf phenotype is restored in *ag clf* double mutants of Arabidopsis (Goodrich et al. 1997). The early flowering and abnormal flower organ phenotypes of type I and II *asBoEMF2.1* broccoli are consistent with the ectopic expression of flower *MADS-box* genes such as broccoli *AG* and *SEP3* (Fig. 3). *SEP3*, a class E flower organ identity gene, enhances the expression of *AP3*, *PI* and *AG* to specify flower organ identity (Immink et al. 2009, Kaufmann et al. 2009) and is involved in flowering time control by its regulation of flowering time genes such as *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *AGAMOUS LIKE 24* (*AGL24*) and *SVP* in the inflorescence (Kaufmann et al. 2009).

The microarray data of the type I and type II *asBoEMF2.1* broccoli might display a mild gene expression change due to a slight reduction of the activities of *BoEMF2*s. The strong phenotype displayed by type III transgenic broccoli is, in principle, comparable with that of the Arabidopsis *emf2* mutant but differs from *emf2* in that the broccoli seedlings produced 3–5 leaves, showed petiole and internode elongation (*asBoEMF2.1-8* in Fig. 2A) and died quickly without producing any flower organs. Limited availability of type III *asBoEMF2.1* plants precluded microarray analysis to elucidate the molecular basis of these phenotypic differences. However, microarray analysis of the type I and type II plants revealed similar as well as divergent functions or gene programs in the two species. For example, 13 flower organ identity genes were misexpressed in the Arabidopsis *emf2* mutant while eight were misexpressed in *asBoEMF2.1* (Table 4, Supplementary Table S2). Ectopic expression of fewer flower organ identity genes may have allowed an abbreviated vegetative development in type III *asBoEMF2.1*, which could not occur in Arabidopsis *emf2*. Unlike *emf2*, *asBoEMF2.1* can produce an elongated internode and petiole. This may be related to the fact that fewer auxin category and *EXPA* family



**Fig. 6** qRT-PCR analysis of gene expression in Rescued *emf2*. Verification of gene expression based on microarray data by qRT-PCR in 7-day-old Arabidopsis seedlings: 35S::*BoEMF2.1* containing transgenic WT (transWT), WT, *emf2* and Rescued *emf2*. The y-axis represents the relative mRNA level of genes normalized to *UBIQUITIN 10* in log value. Genes analyzed are shown on the x-axis. Data are the mean with SD from three independent experiments.

genes, which are involved in hypocotyl elongation (Gray et al. 1998), and cell wall loosening and cell expansion (Sampedro and Cosgrove 2005), respectively, are down-regulated in *asBoEMF2.1* (Supplementary Tables S2, S4). In addition, the death-related genes, *VASCULAR ASSOCIATED DEATH 1* and *RADICAL-INDUCED CELL DEATH 1*, were up-regulated >2-fold in type II *asBoEMF2.1* broccoli, but remained at a normal level in the *Arabidopsis emf2* mutant (Supplementary Table S5). This may be the reason for the early wilting and death of type III *asBoEMF2.1* broccoli.

### BoEMF2.1 restored WT seedling and adult development in *Arabidopsis emf2*

The restoration of nearly 70% genes misexpressed in *emf2* mutants by *BoEMF2.1* (Fig. 5) explained the partial rescue of hypocotyl, petiole and cotyledon phenotypes of *emf2* to that of the WT (Fig. 4). These included the restoration of the genes in auxin and other hormone categories, as well as most of the *EXPA* genes (Table 4, Supplementary Table S4), which are important for cell elongation and expansion in broccoli (Gray et al. 1998, Sampedro and Cosgrove 2005).

*BoEMF2.1* also rescued the expression pattern of flowering time genes, *FT* and *SVP* (Supplementary Tables S3, S4), and flower *MADS-box* genes, thus contributing to the delayed flowering in the Rescued *emf2*. Of the 13 ectopically expressed flower organ identity genes in *emf2*, five were no longer ectopically expressed and, except for *AG* and *SEP3*, the expression of the remaining genes was greatly reduced in the Rescued *emf2* (Table 2, Supplementary Table S4). However, Rescued *emf2* still flowers earlier than does the WT, possibly because of the ectopic expression of *AG* and *SEP3* (Fig. 6), as *SEP3* both activates flower organ identity genes and promotes flowering (Kaufmann et al. 2009). The partial phenotypic and molecular rescue of *emf2* by *asBoEMF2.1* may be attributed in part to the use of a 35S promoter instead of the *EMF2* to drive *BoEMF2.1* expression.

Protein–protein interaction between *EMF2* and *CLF*, which possesses methyl transferase activity, through the conserved 3' VEFS domain in *EMF2* is part of the PRC2 multiprotein complex that mediates gene silencing in *Arabidopsis* (Chanvivattana et al. 2004). Hence, the fact that *BoEMF2.1* restored *EMF2*'s function suggests that *BoEMF2.1* can interact with the *Arabidopsis* *CLF* and participate in the *Arabidopsis* PRC2 action. This is consistent with its 93.7% sequence identity in the VEFS domain to that of *AtEMF2*.

However, unlike *BoAG* and *BoSEP3* in broccoli, *BoEMF2.1* cannot repress the expression of *AG* and *SEP3* in *Arabidopsis*, suggesting that these two genes require a more stringent condition for PRC2-mediated repression than the genes with restored expression. As a result, *BoEMF2.1* cannot fulfill its role in the PRC2 action. The other possibility is that additional proteins, such as *EMF1*, might have evolved in *Arabidopsis* to cooperate with PRC2 in repressing the expression of *AG* and *SEP3*, but not with the PRC2 containing *BoEMF2.1*. Future investigations at the protein structure–function level will address

the question of why *BoEMF2.1* can restore some but not all misexpressed genes in the Rescued *emf2*, thereby elucidating the diverse *EMF2*-mediated repression mechanisms.

## Materials and Methods

### Plant material and growth conditions

Seeds of broccoli (*B. oleracea* var. *italica* cv. Elegance) were purchased from Know-You Seed Company (Kaohsiung, Taiwan), germinated on wet filter paper, transferred to soil in 7 inch pots and grown under a 14/10 h photoperiod at 75–110  $\mu\text{mol m}^{-2} \text{s}^{-1}$  illumination at 20°C.

*Arabidopsis thaliana* cv. Columbia (Col) was used as the WT. The *emf2* mutant constituted the *emf2-1* allele in the *A. thaliana* Col background (Chen et al. 1997). *Arabidopsis* seeds were sown directly in soil or surface-sterilized, placed on a 0.8% agar plate containing half-strength Murashige and Skoog (MS) salt and 1.5% sucrose with or without 20  $\mu\text{g ml}^{-1}$  hygromycin, cold stratified at 4°C for 2–3 d, then transferred to a growth chamber with an 8/16 h photoperiod at 75–150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  illumination at 21°C for germination and development. Fifteen-day-old seedlings grown on an agar plate were transferred into soil and grown under the short-day condition for observation of bolting, flowering and silique development.

### DNA and RNA extraction

For Southern blot analysis, genomic DNA was extracted from the second leaf from the shoot apex of 1-month-old broccoli by use of the Plant Genomic DNA Extraction Kit (Viogene). For gene cloning experiments, genomic DNA was extracted from the broccoli inflorescence shoot.

RNA was extracted from most *Arabidopsis* and broccoli tissue by the TRIzol Reagent method (Invitrogen). To extract RNA from siliques, siliques were ground into powder in liquid nitrogen with use of a mortar and a pestle, and RNA was extracted with the use of extraction buffer (100 mM Tris–HCl, pH 8, 1.4 M NaCl, 20 mM EDTA, pH 8 and 2% cetyltrimethylammonium bromide) as described (Asif et al. 2000). DNA was eliminated by use of the TURBO DNA-free kit (Ambion). The RNA remaining was reverse transcribed by use of the Transcriptor First Strand cDNA Synthesis Kit (Roche) to obtain cDNA products.

### Cloning of broccoli *EMF2* (*BoEMF2*) genes

Genomic DNA and cDNA prepared from the inflorescence of broccoli was used for broccoli *EMF2* gene cloning. We used a combination of chromosome walking and RACE with the BD GenomeWalker Universal Kit (Clontech) and FirstChoice RLM-RACE kit (Ambion), respectively.

Primers designed to clone *BoEMF2* genes are given in Supplementary Table S1.

Cloning began with RT–PCR with the primer pairs for EP1 and EP2 based on the *Arabidopsis EMF2* (*AtEMF2*) sequence to obtain a broccoli cDNA fragment of about 500 nucleotides

(Supplementary Fig. S1). The following EP primers were designed with broccoli sequences obtained during cloning. We used EP3 and EP4 primers designed with the broccoli 500 bp cDNA fragment to obtain the 3' sequence of the cDNA by 3'-RACE and EP5 and EP6 to obtain the 5' sequence of genomic DNA by chromosome walking. With the broccoli genomic DNA sequence (the red part in Supplementary Fig. S1 obtained with EP5 and EP6), we designed EP7 and EP8 primers to generate another cDNA fragment to form a contig with the 3' cDNA sequence obtained with primers EP3 and EP4. Further 5'-RACE was performed with EP9 and EP10 primers to extend the broccoli *EMF2* 5'-cDNA sequence. Finally, EP11 containing a start codon and EP12 containing a stop codon were designed to obtain the full-length coding region of *BoEMF2.1*. However, sequencing results for this full-length *BoEMF2.1* coding region showed sequence contamination, so we searched for another *EMF2*-like sequence (*BoEMF2.2*) (see below).

To clone the *BoEMF2.2* gene, primers EP13 and EP14 specific for a newly identified partial sequence of the *EMF2*-like sequence were designed to obtain a cDNA fragment of about 210 nucleotides (Supplementary Fig. S1). With this short sequence, EP15 paired with EP16 and EP17 paired with EP18 were designed for 3' end and 5' end chromosome walking to obtain the 3' end and 5' end genomic sequences, respectively. Further 3'-RACE was performed with EP13 and EP16 primers to obtain the 3'-cDNA sequence for *BoEMF2.2*. The following primers were used to produce the partially overlapping 5'-cDNA sequences: EP17 and EP19, based on the *BoEMF2.2* genomic sequence; EP11, shared with both *BoEMF2* genes; and EP20, specific to the *BoEMF2.2* genomic sequence (Supplementary Fig. S1). The final 5'-RACE and chromosome walking involved the EP21 and EP22 primers to obtain the 5'-untranslated and 5'-genomic sequences, respectively. A contig of these cDNA and genomic DNA fragment was then reconstructed (Supplementary Fig. S1).

### Southern blot analysis

In total, 10 µg of total DNA was digested with restriction enzymes, separated on a 1.2% agarose gel, blotted onto a nitrocellulose membrane, hybridized with a gene-specific probe and imaged by use of X-ray film. The restriction enzymes were selected with the criterion of no cutting site resides in the gene sequences. The gene-specific probes were labeled with digoxigenin by PCR (Neuhaus and Neuhaus 1993).

### Sequence analysis

All sequences were retrieved from the NCBI (<http://www.ncbi.nlm.nih.gov/>) or TAIR (<http://www.arabidopsis.org/>) database. Sequence analysis involved use of Vector NTI (Invitrogen).

### Plasmid construction, transformation, and screening for transgenic broccoli and rescued *emf2* Arabidopsis seedlings

**Plasmid construction.** The Cauliflower mosaic virus promoter 35S (35S) sequence of *pBI121* was amplified by PCR with the

primers 35S-BaF (TTTGGATCCAGATTAGCCTTTTCAATT) and 35S-SaR (AAAGTCGACCGTGTCTCTCCAAATGA) with *Bam*HI and *Sal*I cutting sites, respectively. The amplified 35S promoter fragment was then introduced into *pCambia1380* vector by use of the *Bam*HI and *Sal*I sites, with selection markers of ampicillin for microbes and hygromycin for plants to create a *pCambia1380-35S-promoter2* vector.

For *BoEMF2* knockdown experiments in broccoli, RNA extracted from broccoli inflorescence was used to synthesize cDNA. Using the cDNA as template, *ABoEMF2-SpF* (AAAGTCAGTATGCCAGGCATTCTCTC) and *ABoEMF2-SaR* (AAAGTCGACTCAATTTGGGAGCTGTTC) with *Spe*I and *Sal*I cutting sites, respectively, were used to clone the full-length CDS of antisense *BoEMF2.1*. The obtained antisense *BoEMF2.1* sequence was subcloned into *pCambia1380-35S-promoter2* vector by use of the *Spe*I and *Sal*I cutting sites to generate a 35S promoter-triggered antisense *BoEMF2.1* expression vector.

For the Arabidopsis *emf2* rescue experiment, primer pairs for *BoEMF2-SaF2* (AAAGTCGACGGGCGATTATGCCAGGCATTCTCTC) and *BoEMF2-SpR* (AAAGTCAGTCAATTTGGGAGCTGTTC) with *Sal*I and *Spe*I cutting sites, respectively, were used to clone the sense *BoEMF2.1* gene from broccoli inflorescence cDNA. The cloned *BoEMF2.1* sequence was then subcloned into *pCambia1380-35S-promoter2* vector by use of the *Sal*I and *Spe*I cutting sites to create a 35S promoter-triggered *BoEMF2.1* expression vector.

**Transformation of antisense *BoEMF2.1* into broccoli.** Cotyledons and hypocotyls of 3-day-old broccoli seedlings were cut into pieces and infected with the *Agrobacterium tumefaciens* strain GV3101 harboring 35S::antisense*BoEMF2.1* as described (Chen et al. 2001). Transgenic broccoli was screened by ability to grow on 20 µg ml<sup>-1</sup> hygromycin and plasmid specific-PCR.

***emf2* rescue by *BoEMF2.1*.** Because homozygous *emf2* is lethal, we introduced 35S::*BoEMF2.1* into heterozygous *emf2* plants, which were identified by allele-specific RFLP genotyping (Calonje et al. 2008) and transformed with *A. tumefaciens* strain GV3101 harboring 35S::*BoEMF2.1* by the floral dipping method (Clough and Bent 1998). T<sub>1</sub> seeds from transformed heterozygous *emf2* plants (T<sub>0</sub>) were surface-sterilized and screened on an agar plate containing 20 µg ml<sup>-1</sup> hygromycin. Hygromycin-resistant seedlings were transplanted into soil to obtain T<sub>2</sub> seeds and plants. T<sub>2</sub> plants homozygous for 35S::*BoEMF2.1* and harboring the Arabidopsis *emf2* mutation were identified by producing 100% hygromycin-resistant progeny and allele-specific RFLP genotyping.

### RT-PCR analysis

The second leaves of 4-week-old antisense *BoEMF2.1* broccoli (*asBoEMF2.1*) and vector-only control were harvested, and the expression of the two *BoEMF2* genes was determined to confirm the knockdown efficiency of the endogenous *BoEMF2* genes (Fig. 3). In total, 2 µg of total RNA was used to synthesize

the first-strand cDNA, and PCR analysis involved primers specific to *BoEMF2.1* and *BoEMF2.2* (**Supplementary Table S1**). The expression of broccoli *actin* (*BoActin*; accession No. AF111812) was a control.

To study gene expression in *asBoEMF2.1* broccoli, 11-day-old seedlings of the *asBoEMF2.1* T<sub>1</sub> generation (G<sub>1</sub>), the T<sub>2</sub> generation (G<sub>2</sub>), vector-only control seedlings and flower and siliques of control plants (**Fig. 4**) were harvested for RNA extraction and cDNA preparation. In total, 2 µg of total RNA was used for first-strand cDNA synthesis. The cDNA was diluted five times in contraction before PCR analysis with gene-specific primers (**Supplementary Table S1**). The PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. Images were obtained by use of a Bio-Rad Doc EQ system, and the intensity of DNA bands was quantified with use of Quantify One (Bio-Rad).

### qRT–CR analysis

For qRT–PCR analysis of *BoEMF2* genes in broccoli, roots and second expanded leaf and stem were harvested from 4-week-old broccoli grown in soil; flower buds and siliques were collected after flowering and silique formation. For Arabidopsis, RNA was extracted from 7-day-old seedlings of the 35S::*BoEMF2.1*-containing WT (transWT), WT, *emf2* and Rescued *emf2*.

Primers for genes were designed for qRT–PCR (**Supplementary Table S1**) with the ABI Prism 7500 sequence detection system (Applied Biosystems) at 58°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative gene expression was determined by the comparative CT method, with the expression of *UBIQUITIN10* (*UBQ10*, At4g05320) used as an internal control for Arabidopsis and *BoActin* for broccoli. The mean value of  $2^{-\Delta CT}$  ( $\Delta C_T = C_T$ , gene of interest –  $C_T$ , control gene) was determined from three independent experiments.

### Microarray experiments and analysis

For broccoli, the seedlings of *asBoEMF2.1* transgenic plants and vector-containing control were grown on half-strength MS agar containing 1.5% sucrose and harvested at 11 d after germination. RNA was extracted by the TRIzol reagent method (Invitrogen), and RT–PCR was performed to determine the endogenous *BoEMF2* level. The *asBoEMF2.1* line with knocked-down expression of *BoEMF2s* and the vector-only control (**Fig. 3**) were used for microarray analysis.

For Arabidopsis, 7-day-old 35S::*BoEMF2.1*-containing WT (transWT) and Rescued *emf2* seedlings grown on hygromycin-containing agar plates were harvested and sorted by cotyledon phenotype: transWT and Rescued *emf2* with round flat and curled outwards cotyledons, respectively (**Fig. 4**). The seedlings of WT and *emf2* grown on MS-only plates were collected and sorted by round flat and small oval shaped cotyledons, respectively (**Fig. 4**). After confirming the genotype by allele-specific RFLP (Calonje et al. 2008) and *BoEMF2.1* expression (**Supplementary Fig. S2**), microarray analysis was performed.

For microarray experiments, 10 µg of total RNA was used as a template for cDNA synthesis, cRNA was biotin labeled by in vitro transcription then fragmentation by use of the GeneChip Expression Analysis Technical Manual rev5 (Affymetrix). In total, 10 µg of labeled cRNA was hybridized to the 22 K ATH1 GeneChip (Redman et al. 2004) at 45°C for 16.5 h. Washing and staining involved use of Fluidic Station-450, and genechips were scanned by use of the Affymetrix GeneChip Scanner 7 G. The mean gene expression indices for all chips were scaled to an arbitrary target of 500.

For broccoli gene expression analysis, only genes with hybridization signals  $\geq 60$  in at least one of the two samples, the vector-only and the *asBoEMF2.1* broccoli, were analyzed. For Arabidopsis, only genes with hybridization signals  $\geq 50$  in at least one of four plant samples, *emf2*, WT, Rescued *emf2* and transWT, were analyzed. The hybridization signal ratio of fold change of gene expression in broccoli was analyzed by the ratio of *asBoEMF2.1* to vector-only control, with a ratio  $\geq 2$  or  $\leq 0.5$  used to identify up- or down-regulated genes, respectively. The fold change of gene expression in Arabidopsis was based on the ratio of *emf2* to the WT over that of Rescued *emf2* to transWT, with a ratio  $\geq 2$  or  $\leq 0.5$  used to identify up- or down-regulated genes, respectively.

### Supplementary data

**Supplementary data** are available at PCP online.

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